instructions

product code:

**E79000M**

# DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE

**Warning**
*For research use only.*
Not recommended or intended for the diagnosis of disease in humans or animals.
Do not use internally or externally in humans or animals.

ⓘ 74000020 Ed. AC, 1999-01

Amersham Biosciences

## Handling

**Storage**
Store at -15 ˚C to -30 ˚C.

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## Quality control

All batches of DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE are assayed according to the protocols described in this booklet. Reactions are run on MegaBACE 1 000 sequencing instrument. Specifications for release are based on assessment of sequence by length of read, accuracy and signal quality with > 500 bases of readable sequence achieved.

## Materials not supplied

**Reagents**

- **Water**—Use only deionized, distilled water for the sequencing reactions.
- **Sequencing primers**—The following DYEnamic ET primers have been formulated specifically for use with

## Components of the kit

Solutions included in DYEnamic™ ET Primer DNA Sequencing Reagents for MegaBACE™ have been carefully formulated for optimal sequencing results. Each reagent has been tested extensively and its concentration determined to meet Amersham Biosciences standards. It is strongly recommended that all reagents supplied in the kit be used as described in this protocol.

The following components are included in this product:

Thermo Sequenase™ (32 U/µl)—MegaBACE formulation (E79000M)

MegaBACE reaction buffer (260 mM Tris, 32.5 mM $MgCl_2$, pH 9.5) (US79607)

Loading solution (70% formamide, 1 mM EDTA) (US79916)

The following reagents should be obtained for use with this protocol:

MegaBACE dG sequencing blend (US79655)—contains the following four termination mixes in separate vials:
A termination mix (US79586); C termination mix (US79587); G termination mix (US79588); T termination mix (US79589)

OR

MegaBACE 7-deaza-dGTP sequencing blend (US79657)—contains the following four termination mixes in separate vials:
A termination mix (US79590); C termination mix (US79591); G termination mix (US79592); T termination mix (US79593)

## Materials not supplied *(continued)*

MegaBACE 1 000 sequencing instrument:

US79475 MegaBACE -40 ET Forward Primer Set
GTT TTC CCA GTC ACG ACG

US79880 MegaBACE -21 M13 ET Forward Primer Set
TGT AAA ACG ACG GCC AGT

US79970 MegaBACE -28 M13 Reverse 1 ET Primer Set
AGG AAA CAG CT*A TGA CCA T

US79890 MegaBACE -28 M13 Reverse 2 ET Primer Set
AGG AAA CAG CT*A TGA CAT G

US79900 MegaBACE -29 M13 Reverse ET Primer Set
TCA GGA AAC AGC TAT GAC C

US79850 MegaBACE SP6 ET Forward Primer Set
ATT TAG GTG ACA CTA TAG

US79855 MegaBACE T7 ET Forward Primer Set
TAA TAC GAC TCA CTA TAG GG

- **7.5 M ammonium acetate**
- **Ethanol (100% and 70%)**—For sample preparation for injection.

## Components of the kit *(continued)*

The reagents comprising this product should be stored at -15 ˚C to -30 ˚C (NOT in a frost-free freezer). All reagents should be kept on ice when removed from storage for use. For convenience, the kit can be stored at 2–4 ˚C for up to three months with no loss of performance; however, this should be avoided if the reagents will not be completely consumed within three months.

## World wide web address

**http://www.amershambiosciences.com**

Visit the Amersham Biosciences home page for regularly updated product information.

## Materials not supplied *(continued)*

- **Electrophoresis matrix**—Long-read Matrix (US79676) for capillary electrophoresis. This is linear polyacrylamide or LPA.

**Equipment**

- **Liquid handling supplies**—Vials, pipettes, centrifuge and vacuum centrifuge. All sequencing reactions should be run in plastic microcentrifuge tubes (typically 0.5 ml) or 96-well plates suitable for thermal cycling.
- **Instrument**—This kit is designed for optimal performance with MegaBACE 1 000 sequencing instrument.
- **Thermal cycler**—For thermally cycled incubations between 50 ˚C and 95 ˚C (1–100 cycles).

## Safety warnings and precautions

***Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.***

All chemicals should be considered as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle this product. Suitable protective clothing such as laboratory overalls, safety glasses and gloves should be worn. Care should be taken to avoid contact with the skin or eyes; if contact should occur, wash immediately with water (see Material Safety Data Sheet for specific recommendations).

**Warning:** This protocol requires the use of ethanol, a flammable liquid. Gel reagents may contain acrylamide, a neurotoxin and suspected carcinogen. Please follow the manufacturer's Material Safety Data Sheet regarding safe handling and use of these materials.

## Introduction

DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE are formulated for use with DYEnamic ET Primers and MegaBACE 1 000, a powerful capillary-based sequencing system designed to provide high throughput sequencing laboratories with unprecedented read-lengths, high pass rates and industry-leading data quality.

The reagents in the kit are formulated for use with sequencing mixes containing either dGTP or 7-deaza-dGTP for resolution of band compressions. For sequencing, a mixture comprised of Thermo Sequenase DNA polymerase, Reaction buffer, A, C, G or T termination mix, template DNA and dye-labelled primer is thermally cycled. The reaction products are then precipitated with salt and ethanol and resuspended in a formamide loading solution for separation and detection using MegaBACE 1 000 sequencing instrument.

This protocol provides the methods necessary for sequencing with DYEnamic ET Primers and DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE.

**Capillary array electrophoresis**

In capilllary array electrophoresis, narrow-bore capillaries filled with a separation matrix are used to resolve DNA sequencing fragments. Under the force of an electric field, the fragments migrate into (electrokinetic injection) and through (electrophoresis) the capillaries and are separated according to size. Because of their large surface-to-volume ratio, capillaries allow efficient removal of heat generated during electrophoresis. Since higher voltages can be used, fragments migrate more rapidly and separation times are shorter.

The MegaBACE system uses six arrays of 16 capillaries each to provide rapid parallel separation of dye-labelled DNA fragments from a total of 96 samples. The capillaries are filled with a replaceable, non-crosslinked linear polyacrylamide (LPA) matrix. By applying a variable time and variable voltage pulse, the instrument can electrokinetically inject all 96 samples from a 96-well plate simultaneously. Each capillary has a clear detection window located at a fixed distance from the sample loading point. During electrophoresis, the instrument uses laser excitation and a patented confocal optical detection system to both excite and detect the dye-labelled DNA fragments as they migrate past the window (1-8).

**Energy transfer dye primers**

Energy transfer (ET) dye primers are uniquely designed oligonucleotides with two fluorescent dyes attached to each primer molecule for improved detection (9). The donor dye absorbs light of the wavelength of the argon ion laser in the sequencing instrument and transfers this absorbed light energy to the acceptor dye. The acceptor dye then emits the absorbed energy as fluorescence at its characteristic emission wavelength for detection by the sequencing instrument. Since ET primers all absorb the laser light efficiently, their effective fluorescence intensity is 2–12 times greater than primers with single-dye labels.

DYEnamic ET Primers developed for MegaBACE use 5-carboxyfluorescein (FAM) as the donor dye and rhodamine 110 (R110) (C), 6-carboxyrhodamine (R6G, also known as REG) (A), N,N,N',N'-tetramethyl-5-carboxyrhodamine (TMR, also known as TAMRA) (G) and 5-carboxy-X-rhodamine (ROX) (T) as the acceptor dyes.

**Thermo Sequenase DNA polymerase**

Thermo Sequenase is an exonuclease-free, thermostable DNA polymerase specifically engineered by Amersham Biosciences for cycle sequencing. The enzyme readily accepts dideoxynucleotide terminators (10) and generates bands of uniform intensity, much like T7 Sequenase™ DNA polymerase (11, 12). These characteristics, along with its thermostability, make it ideal for use in cycle sequencing and greatly facilitate data interpretation and accurate base-calling using automated sequencing software.

The formulation of the enzyme includes thermostable inorganic pyrophosphatase (TAP) cloned from *Thermoplasma acidophilum*. TAP hydrolyzes the inorganic pyrophosphate product of nucleotide polymerization, thus preventing the occurrence of pyrophosphorolysis and subsequent loss of peaks from sequence data.

**Cycle sequencing**

In traditional dideoxynucleotide sequencing, synthesis of a new strand of DNA is initiated at a specific priming site by a DNA polymerase in the presence of deoxynucleotide triphosphates (dNTPs). Extension of the strand continues until a chain-terminating dideoxynucleoside triphosphate (ddNTP) is incorporated (13). By controlling the relative concentrations of dNTPs and ddNTPs, the majority of the DNA fragments generated will terminate at lengths within the resolving capacity of the gel matrix used for separation. When the fragments are labelled

with dyes, fluorescent sequencing instruments can derive sequence information from the fluorescence emitted by the fragments as they move through a particular area of the gel.

When thermostable DNA polymerases are used, sequencing reactions can be cycled through alternating periods of thermal denaturation, primer annealing and extension/termination to increase the signal levels generated from template DNA (14–19). For each cycle, the amount of sequencing product available for analysis will be equivalent to the amount of primed template, with the theoretical yield roughly equal to the number of cycles (in practice, each cycle is not 100% efficient and the amplification will be somewhat lower). By using a thermostable enzyme such as Thermo Sequenase DNA polymerase, programmed cycling can be performed with no need for additional enzyme. A cycling protocol is especially useful when the amount of template is limiting or the sensitivity of the detection system is low.

The use of DYEnamic ET Primers and Thermo Sequenase DNA polymerase in conjunction with a cycle sequencing format yields the highest quality sequence data with minimal reagent, template and labor requirements.

## Protocols

**Preliminary preparations and general handling instructions**

A thermocycler should be pre-programmed as described on page 11. For additional information concerning cycling parameters, see Appendix 2 on page 15.

The following reagents should be thawed on ice prior to use:

- DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE (enzyme, buffer and loading solution)
- MegaBACE termination mixes (either MegaBACE dG sequencing blend or MegaBACE 7-deaza-dGTP sequencing blend)
- DYEnamic ET Primer Set for MegaBACE
- Template DNA

Whenever possible, tubes should be kept capped and on ice to minimize evaporation of the small volumes of reagents used. Reagents should be dispensed using disposable-tip micropipettes, and care should be taken to avoid contamination of stock solutions. Reaction mixtures must be thoroughly mixed after each addition, typically by "pumping" the solution two or three times with a micropipettor without creating air bubbles. Tubes/plates should be centrifuged briefly to collect the reaction mixtures at the bottoms of the tubes/wells. A final brief centrifugation step should be performed to remove any bubbles prior to injection into the sequencing instrument.

### 1 Sequencing with DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE

This protocol is a recommended starting point. Further experiments to optimize the amount of primer and template DNA and conditions for thermal cycling may be required. If changes in reaction volume are necessary, the volume of reaction buffer used in the termination reactions should be adjusted. Optimal results are obtained when the final $MgCl_2$ concentration in each termination reaction is ~ 2–4 mM.

**1.1** Dilute the DNA template in deionized water or in buffer containing no more than 0.1 mM EDTA. The recommended quantities of template DNA are 100–500 ng (40–200 fmol) of single-stranded M13 DNA or 200–800 ng (80–400 fmol) of double-stranded DNA.

For additional information concerning preparation of DNA templates, see Appendix 1 on page 14.

## 2 Preparation of master mixes

**2.1** For each template to be sequenced, prepare the following master mixes by combining an A, C, G or T termination mix (from either MegaBACE dG sequencing blend or MegaBACE 7-deaza-dGTP sequencing blend) with the appropriate primer. When all the reagents have been dispensed, mix thoroughly by gentle pipetting. The table below shows volumes for one sequencing reaction. Adjust the volumes accordingly for additional reactions.

| | Master Mixes | | | |
|---|---|---|---|---|
| | **A** | **C** | **G** | **T** |
| **Reaction buffer** | 0.64 µl | 0.64 µl | 0.64 µl | 0.64 µl |
| **ET Primer R6G (0.5 µM)** | 1.0 µl | | | |
| **ET Primer R110 (0.5 µM)** | | 1.0 µl | | |
| **ET Primer TMR (1.0 µM)** | | | 1.0 µl | |
| **ET Primer ROX (1.0 µM)** | | | | 1.0 µl |
| **Termination mix** | 1.0 µl ddA | 1.0 µl ddC | 1.0 µl ddG | 1.0 µl ddT |
| **Super Q water** | 2.26 µl | 2.26 µl | 2.26 µl | 2.26 µl |
| **Thermo Sequenase (32 U/µl)** | 0.1 µl | 0.1 µl | 0.1 µl | 0.1 µl |
| **Total volume** | 5.0 µl | 5.0 µl | 5.0 µl | 5.0 µl |

## 3 Preparation of termination reactions

**3.1** Transfer 5 µl of each termination master mix prepared in step 2.1 to a clean tube/well. Add 5 µl of DNA template in water to each tube/well for a final termination reaction volume of 10 µl. Mix thoroughly by gentle pipetting.

**3.2** After preparing the termination reactions, cap the tubes or seal the plates and place the reactions into the pre-programmed thermal cycler.

**3.3** Start the cycling program. The cycle parameters shown below are general guidelines. Specific cycling parameters used will depend on the amount and purity of the template DNA. For additional information concerning cycling parameters, see Appendix 2 on page 15.

95 ˚C, 15 s
50 ˚C, 15 s
70 ˚C, 1 min
Repeat for 20–30 cycles.

**3.4** After cycling is complete, centrifuge the tubes/plate briefly to collect the reaction mixtures at the bottoms of the tubes/wells.

## 4 Post-reaction clean-up—Preparation of sequencing samples for capillary injection

**4.1** Pool the four termination reactions into a single tube.

**4.2** Optional step: To improve the efficiency of ethanol precipitation, a carrier such as glycogen (10–20 µg) may be added to the pooled termination reactions.

**4.3** Add 0.1 volume of 7.5 M ammonium acetate and 3 volumes of 100% ethanol to the pooled samples and mix well. Place in a freezer for 15 min or overnight.

**4.4** Centrifuge the tubes in a microcentrifuge (~ 12 000 rpm) for 30 min at either room temperature or 4 °C. Centrifuge 96-well plates for at least 30 min at 2 500 × g.

**Note:** If plates are centrifuged at a lower relative centrifugal force, the time of centrifugation must be increased. Samples can routinely be centrifuged for 30 min at 3 100 × g.

**4.5** Remove the supernatant by aspiration. Wash the pellet with 200 µl of 70% ethanol. Vortex briefly and centrifuge as described above.

**4.6** Remove the supernatant by aspiration and air-dry the DNA pellets for 10–15 min. Alternatively, the samples can be dried in a vacuum centrifuge for 2–3 min. Do **NOT** over-dry the DNA pellets.

**4.7** Dissolve each pellet in 10 µl of Loading solution and vortex vigorously for 10–20 s to ensure complete resuspension. Briefly centrifuge to collect the samples at the bottoms of the tubes/wells and to remove any bubbles.

**Note:** The DNA pellet MUST be completely dissolved at this step for optimal sequencing results. If a fixed angle rotor was used for centrifugation, the DNA pellet will be on the side of the microcentrifuge tube. This material must be washed to the bottom of the tube to ensure that the entire reaction product is loaded onto the gel.

The following chart presents a brief summary of the steps used in preparing samples for capillary injection:

| | |
|---|---|
| Pool termination reactions | 40 µl |
| Glycogen (10 µg/µl) | 2 µl |
| 7.5 M ammonium acetate | 4 µl |
| 100% ethanol | 140 µl (or ~ 3 volumes) |
| Freezer 15 min to overnight | |
| Microcentrifuge 30 min | |
| Remove supernatant | |
| 70% ethanol wash | 200 µl |
| Microcentrifuge 15–30 min | |
| Remove supernatant | |
| Air-dry or speed vac spin 5 min | |
| Loading solution (resuspension) | 10 µl per reaction |

## 5 Instrument setup and analysis of data

**5.1** For instrument setup and data analysis, please refer to the instrument documentation supplied with the MegaBACE 1 000 sequencing instrument.

**5.2** Verify that the MegaBACE 1 000 instrument has been equipped with the following standard filter set for blue dye primer sequencing:

| | |
|---|---|
| Beam splitters | A: 540DRLP<br>B: 600DRLP |
| Bandpass filters | 555DF20 (Channel 1)<br>525DF15 (Channel 2)<br>620LP (Channel 3)<br>585DF20 (Channel 4) |

**5.3** Set the instrument for a dye primer run with the parameters suggested below. For additional information concerning injection and run parameters, see Appendix 3 on page 16.

**Electrophoresis Parameters**
Sample injection voltage: 3 kV
Sample injection time: 40 s
Run voltage: 9 kV
Run time: 100 min
Temperature: 44 °C

**Fill/Flush Parameters**
High-pressue time: 200 s
Relaxation time: 20 min
Pre-run time: 5 min
Pre-run voltage: 9 kV
Matrix flush time 1: 16 s
Matrix flush time 2: 7 s
Low-pressure time: 240 s

**Optics Parameters**
PMT 1: 750 volts or as set
PMT 2: 750 volts or as set
Laser mode: Blue
Filter 1: 555DF20
Filter 2: 525DF15
Filter 3: 620LP
Filter 4: 585DF20
Beam splitter A: 540DRLP
Beam splitter B: 600DRLP

## Appendix 1:
## Preparation of template DNA—general considerations

Preparation of highly pure DNA template is the single-most important factor affecting the quality of sequencing data produced with MegaBACE 1 000 sequencing system and the success rate achieved. Template of suitable quality can be prepared using a variety of procedures and commercial products.

**Preparation of single-stranded plasmid DNA**
Several published methods are available for preparing single-stranded DNA from clones in M13 vectors and hybrid plasmid-phage (phagemid) vectors (20, 21).

**Preparation of double-stranded PCR† product templates**
PCR products are typically very good sequencing substrates for the MegaBACE system since their concentrations are relatively uniform, and they are relatively free of inhibitory contaminants and large amounts of unsequenced template DNA (e.g. vector sequence) that can interfere with injection. For removal of PCR primers and nucleotides, samples can either be diluted or treated with exonuclease I and shrimp alkaline phosphatase using the PCR Product Pre-sequencing Reagent Pack (US70995).

**Preparation of double-stranded plasmid DNA**
Sequencing double-stranded templates with DYEnamic ET Primer DNA Sequencing Reagents for MegaBACE requires no changes in the protocol. Alkaline denaturation is not required for these templates. For optimal results, plasmid DNA should be purified for use. Cesium chloride gradients, polyethylene glycol (PEG) precipitation, adsorption to glass, columns, and other common DNA purification methods all produce

DNA suitable for use in this system. Although there are many popular protocols for purifying plasmid DNA from 2 ml to 10 ml cultures, we have had consistent success using Qiagen,™ boiling (22, 23) and alkaline (24) mini-prep methods.

## Appendix 2: Cycling parameters and quantity of template

The temperatures used for cycling the sequencing reactions are based on characteristics of the sequencing primer whereas the number of cycles required will depend on the quantity and quality of the template DNA. The following guidelines should assist in choosing cycling parameters.

**Cycling temperatures**

For a typical sequencing primer of 15–25 bases and a G/C content of ~ 50%, the melting temperature will generally be 50–65 °C. We suggest using a three-temperature cycling program—denaturation at 95 °C for no more than 15 s; annealing at 45–55 °C for 5–15 s; and extension at 70–72 °C for 30–120 s. The duration of neither the annealing nor denaturation steps appears to be critical, and even brief pauses at these temperatures seem to be effective. If in doubt, a wide temperature range should be chosen with brief pauses at the temperature extremes.

Although some users include an initial denaturation step at 95–98 °C, this should be avoided since Thermo Sequenase DNA polymerase rapidly denatures during extended periods at high temperatures.

**Number of cycles and quantity of template**

The number of cycles required for optimal results depends on both the amount (fmol) and purity of template DNA used as well as on the sensitivity of the fluorescent detection method. Using the protocol described in this booklet, the minimum quantities of highly purified DNA that

have been successfully sequenced are ~ 25 fmol (50 ng) of M13mp18 DNA and ~ 50 fmol (100 ng) of pUC18 DNA.

For routine sequencing, at least 50 fmol (~ 100 ng) of M13 or 150 fmol (~ 300 ng) of plasmid DNA is sufficient. When small quantities of template DNA are used, signal intensity is strongly influenced by the number of cycles. If template quantities are near the limits stated above, the number of cycles should be increased. Usually 20–30 cycles are adequate for good signal generation. As noted in the Troubleshooting section, if too much DNA (> 5 pmol) is used, short read-lengths are likely.

## Appendix 3: Injection and run parameters

The quality of an electrokinetic injection is affected by a number of factors including the amount of template DNA, the presence of excess salts, the consistency of the samples within the plate, the loading solution, the electrophoresis run temperature, and the nature of the separation matrix being used. Controlling these variables is the best way to optimize the results from a capillary electrophoresis system. Although general guidelines are provided below, injection protocols should be optimized for each sample.

**Injection solution, voltage and duration**

The ionic strength of the solution used to dissolve DNA sequencing samples can dramatically affect the electrokinetic injection parameters. A broad range of injection parameters can be used with samples dissolved in formamide loading solutions. For these samples, baseline injection conditions of 2 kV for 60 s are an appropriate starting point. If signals are weak and read-lengths are short, the duration of injection can be increased. If data lack resolution due to template overloading,

the resuspension volume can be increased or the duration of injection can be reduced (see Troubleshooting section). The longest read-lengths are obtained when the product of the time and the voltage of injection ranges from 50 to 200 kV s. For the greatest reproducibility, the applied injection voltage should not exceed 5 kV.

Sequencing samples may also be dissolved in water for injection. If spin columns or gel filtration plates are used rather than salt/ethanol precipitation, it is convenient to leave the samples in the eluent and directly inject from water. However, the range of voltage and duration of injection used with samples injected from water is typically narrower. A good starting point for injection from water is 2 kV for 20 s with optimal results typically obtained between 25 and 50 kV s. For samples containing smaller amounts (< 50 ng) of template DNA, both signal intensity and base-calling accuracy can be improved by resuspending sequencing products in water. However, for customers with variable amounts of DNA in their sequencing reactions, resuspending samples in formamide loading solutions may allow the use of a broader range of injection conditions.

**Injection volume**

According to the protocol outlined in this booklet, the dried DNA pellet from a sequencing sample should be dissolved in 10 µl of formamide loading solution. The minimum volume for sample resuspension and injection is ~ 5 µl. If volumes < 5 µl are used, contact of both the electrode and the capillary with the sample is likely to be inconsistent. Larger resuspension volumes (10–30 µl) can improve the results observed with samples containing an excess amount of template DNA by reducing over-injection of sample into the capillary.

**Run parameters**

Using standard run conditions of 100 min at 9 kV, average read-lengths > 500 bases with 98.5% accuracy can be expected with control templates. These conditions allow for an instrument turn-around time of ~ 2 h. To take advantage of the superior resolving power of the LPA matrix and to achieve the longest read-lengths, electrophoresis should be carried out for 200 min at 6 kV. Even with these recommendations, the quality and quantity of the template remain the most important factors affecting read-length and success rate.

## Troubleshooting

Prior to diagnosing problems associated with the sequencing reaction chemistry, operation of the MegaBACE instrument should be verified for optimal performance by injecting a plate of MegaBACE M13 DNA Sequencing Standards (US79678) and carrying out electrophoresis according to the accompanying protocol. If the average overall read-length of this standard plate is < 500 bases (98.5% accuracy), the instrument may require some routine maintenance such as capillary cleaning or focusing. For further details, please contact the Molecular Dynamics Field Support office at 1-800-743-7782.

**Problem: Sequencing signals are weak.**

Weak signal strength occurs when insufficient dye-labelled DNA sequencing product is injected into a capillary. Although it may be the result of unsuccessful sequencing, it may also be due to inefficient injection of the reaction products. It is important to remember that electrokinetic injection into capillaries is more efficient if the ionic strength of the loading solution is low.

*Possible causes/solutions*

1. The ionic strength of the loading solution was too high. Electrokinetic injection into capillaries is more efficient if the ionic strength of the loading solution is low.
2. The duration of electrokinetic injection was too short. Confirm that the recommended injection conditions were used. Change the injection conditions by increasing the duration of injection 50–100%.
3. There was residual salt in the samples. This can affect the ionic strength of the sample and inferfere with electrokinetic injection.

Confirm that the recommended salt was used for ethanol precipitation and that a wash step was performed. If products were prepared using spin columns, confirm that they were eluted in water. Some preparations of size exclusion chromatography media are pre-swollen in a salt-containing buffer and must be washed several times with water to remove the salt. In some cases, it may be necessary to wash the dry media several times to remove residual ions that may interfere with injection. Alternatively, sequencing reaction pellets may be resuspended in water rather than formamide. See Appendix 3 for additional information concerning guidelines for electrokinetic injection.

4. The template DNA contained excess EDTA. Resuspend the template DNA in water or in dilute buffer containing < 0.1 mM EDTA.

5. The template DNA was of poor quality. Contaminants carried over with template preparations can precipitate and interfere with the efficiency of electrokinetic injection. There is a strong correlation between the quality of template DNA and the success of sequencing experiments.

6. Either the quantity of template DNA or the number of cycles used for amplification was insufficient. Increase either the amount of DNA used in the reaction or the number of cycles.

7. The annealing temperature was incorrect for the primer being used. Check the primer melting temperature and adjust the annealing temperature accordingly.

8. Too little primer was used. The total recommended amount of primer is 3 pmol (0.5 pmol each of the R6G- and R110-labelled primers, 1 pmol each of the TMR- and ROX-labelled primers).

Using < 1 pmol of total primer will generally result in reactions with weak signal strength.

9. The volumes of the reagents used were incorrect. Check for the correct volumes of the reagents to be added.

10. Reagent integrity was compromised. Repeat the reaction using a control DNA to verify reagent integrity.

**Problem: Signal (color) in one or more of the channels is weak or non-existent.**

Absence of signal can result from incorrect instrument setup, failure to combine or mix reagents properly prior to performing the sequencing reactions, or loss of sequencing products during ethanol precipitation.

*Possible causes/solutions*

1. The beam splitters and bandpass filters were not installed in the correct orientation. Check for correct installation.

2. One or more reaction components were omitted from the reaction mix. Verify that all reaction components (primer, buffer, nucleotides, enzyme and template DNA) have been added to all termination reactions.

3. DNA sequencing products were not pooled or were precipitated improperly. Follow the protocol exactly as described.

**Problem: No signal above background is observed after electrokinetic injection.**

Failure to observe any signal above background in all four channels can result from several factors related to either the sequencing chemistry or the instrument. For instrument problems, consult the MegaBACE

User's Manual and call the Molecular Dynamics Field Support office at 1-800-743-7782 for further details.

*Possible causes/solutions*

1. The buffer plate rather than the sample plate was used for injection. Repeat the injection using the sample plate.
2. One or more reaction components were omitted from the reaction mix. Verify that all reaction components (primer, buffer, nucleotides, enzyme and template DNA) have been added to all termination reactions.

**Problem: Late starts and broad, poorly resolved peaks are prevalent in the sequences.**

Late appearance of the primer peak and poorly resolved sequencing fragment peaks are most often due to capillary overloading as the result of too much template DNA entering during electrokinetic injection. Template DNA molecules compete with sequencing products for injection, and overabundance of these large DNA molecules can cause a reduced or sudden drop in the capillary current. Overloaded samples frequently have low signals since the peaks are broad and diffuse, and it is common to misdiagnose overloading as insufficient signal. Under optimal conditions, detection of all samples should begin within a few minutes of each other.

*Possible causes/solutions*

1. Too much template DNA was used during electrokinetic injection causing overloading of the capillary. Repeat the sequencing reactions using less template. To determine the optimal amount of

template to use, perform a titration of the template over a 50-fold range (0.2, 0.5, 1, 2, 5, and 11 µl, for example).

2. The injection conditions were not optimal. Confirm that the recommended injection conditions were used. Repeat the injection of the samples for a shorter duration (25–50% shorter).
3. The injection voltage was too high. Reduce the voltage by 25–50%.
4. Too little loading solution was used. Increase the volume of the resuspended samples by 100–200% and re-inject using the original parameters.
5. The electrokinetic injection and voltage parameters used were not optimal for injecting samples from water. If injecting from water, evaporate the samples to dryness and inject from loading solution.

**Problem: Peak spacing changes during the run giving rise to the "accordion effect."**

Occasionally the current in a capillary will suddenly drop or undergo rapid fluctuations resulting in dramatic changes in the peak spacing.

*Possible causes/solutions*

1. Samples were near the limits of overloading. Follow the suggestions for overloading described above.
2. Bubbles formed in the LPA matrix either during injection or during electrophoresis. Repeat the injection.

**Problem: Extensions appear short with read-lengths limited to < 350 bases.**

*Possible causes/solutions*

1. Too much template DNA was used in the sequencing reaction. In some cases, the use of too much template, especially PCR product DNA, can exhaust the supply of ddNTPs in the reaction. If this occurs, the sequence will suddenly fade before reaching 350 bases in length. This problem is especially prevalent if excess primer is also used. Use < 1 pmol of template DNA and 5 pmol of primer for each sequence. By using less template, the concentration of any potential contaminants is also reduced.
2. The extension step was too short. Increase the time of the extension step in the cycling program to 2–4 min.

**Problem: Localized, broad four-color peaks are common in the sequence.**

The appearance of large, broad four-color peaks extending off-scale may result from amplification of DNA that occurs during the cycling process. This is similar or identical to the formation of primer-dimer bands and other artifacts often seen in PCR, but in this case, products are made with labelled primers. These artifacts are distinguishable from stops in the sequence primarily by their magnitude.

*Possible causes/solutions*

1. The annealing temperature was too low. Use more stringent annealing conditions when cycling. Increasing the annealing temperature from 50 ˚C to 55–57 ˚C is often effective.
2. Too many cycles were used. Use fewer total cycles. Generally, 20–30 cycles are sufficient for effective generation of sequencing

products. Increasing the number of cycles beyond this range may encourage the formation of cycling artifacts.

3. The concentration of primer was too high. Reduce the concentration and repeat the procedure.

4. Self-annealing of the primer occurred. In some cases, the only effective solution is to choose a different primer.

**Problem: Localized broad single-color peaks are prevalent in the sequences.**

The appearance of large, broad single-color peaks in dye primer sequencing products may result from hydrolysis of the dye primer, especially when a long pre-denature cycling step (> 95 ˚C for > 30 s) is used. The rationale for including a long pre-denature step is to completely denature double-stranded DNA molecules, thus increasing the amount of template strand available for annealing and extension. In practice, however, this affords little if any improvement for plasmid sequencing, and in fact only serves to hydrolyze the dyes on the primer and denature the DNA polymerase.

*Possible causes/solutions*

1. The dye primers were hydrolyzed during a pre-denature step. Do not denature at temperatures > 95 ˚C or for periods longer than 30 s.

If problems persist, please contact Amersham Biosciences Technical Service for assistance.

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T7 Sequenase DNA polymerase—This reagent (kit) is covered by or suitable for use under one or more US Patent numbers: 4,795,699; 4,946,786; 4,942,130; 4,962,020; 4,994,372; 5,145,776; 5,173,411; 5,266,466, 5,409,811, 5,498,523 and 5,639,608. Patents pending in US and other countries.

*Exo* I and Shrimp Alkaline Phosphatase—US Patent 5,756,285.

Energy Transfer dyes and primers—US Patent numbers: 5,654,419, 6,688,648, and 5,707,804.

Thermo Sequenase DNA polymerase—Patent pending.

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